PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/11, 15/16, 15/18, C07K 14/475, 14/51, 14/47, 16/00, 16/22, A61K 38/16, 38/18

A1

(11) International Publication Number: **WO 97/00318**

(43) International Publication Date: 3 January 1997 (03.01.97)

(21) International Application Number: PCT/US96/09127

(22) International Filing Date: 4 June 1996 (04.06.96)

(30) Priority Data:
08/474,578 7 June 1995 (07.06.95) US

(71) Applicant: OSTEOSA INC. [US/US]; Suite 201, 2040 Babcock Road, San Antonio, TX 78229 (US).

(72) Inventors: ROODMAN, G., David; 13271 Hunter's View, San Antonio, TX 78230 (US). REGGY, Sakamuri, V.; 7314 Oak Manor Drive #314, San Antonio, TX 78229 (US). MUNDY, Gregory, R.; 3719 Morgan's Creek, San Antonio, TX 78230 (US).

(74) Agents: STEINHARDT, Paul, C. et al.; Campbell & Flores L.L.P., Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: OSTEOCLAST GROWTH REGULATORY FACTOR

(57) Abstract

The present invention relates to a novel osteoclast growth regulatory factor, "osteoclast stimulating factor (OSF)", which stimulates the growth and/or differentiation of osteoclast cells, methods of preparing OSF, therapeutic and diagnostic uses thereof, nucleic acid sequences encoding all or part of OSF, antibodies to and antagonists of OSF, and assays for OSF, and provides therapeutics for the prevention and treatment of diseases involving bone tissue including osteoporosis, Paget's disease, and osteopetrosis.

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OSTEOCLAST GROWTH REGULATORY FACTOR

FIELD OF THE INVENTION

The present invention relates to a novel osteoclast growth regulatory factor, "osteoclast stimulating factor (OSF)," which stimulates the growth and/or differentiation of osteoclast cells, methods of preparing OSF, therapeutic and diagnostic uses thereof, nucleic acid sequences encoding all or part of OSF, antibodies to and antagonists of OSF, and assays for OSF, and provides therapeutics for the prevention and treatment of diseases involving bone tissue including osteoporosis, Paget's disease, and osteopetrosis.

Living bone tissue is continuously being replenished by the process of resorption and deposition of calcium minerals. This process, described as the absorption-resorption cycle, is facilitated by means of substantially two cell types, the osteoblasts and the osteoclasts. The osteoclast is a multinucleated cell and is the only cell in the body known to have the capacity to degrade (or resorb) bone. This resorption activity is accomplished by the osteoclast forming pits (resorption lacunae) in bone tissue, and, in fact, osteoclast activity in cell culture is measured by their capacity to form these pits on slices of mineralized tissue such as bone or sperm whale dentine. The osteoclast is derived from a hematopoietic precursor which it shares with the formed elements of the blood (Mundy & Roodman, In: Bone and Mineral Research V, Elsevier, Peck WA (ed.)., Chapter 5, pp. 209-280, 1987). The precursor for the osteoclast is a mononuclear cell (cell with a single nucleus) which is found in the bone marrow and which forms the mature and unique multinucleated osteoclast after undergoing replication and differentiation by means of cell fusion. The mature osteoclast is distinguished from other multinucleated cells by the presence of the enzyme tartrate-resistant acid phosphatase (TRAP) which is used as an osteoclast cell marker.

Cells found in blood and bone respond to specific protein factors excreted by other cells in response to various stimuli. These factors are referred to as cytokines, many of which have been identified by their biological characteristics and their unique amino acid sequences. Each cytokine presents a unique spectrum of characteristics utilized to distinguish each specific cytokine from others. Certain cytokines stimulate the growth and/or differentiation of specific types of cells, while other cytokines target cancerous cells for destruction. Exemplary cytokines include granulocyte colony stimulating factor (G-CSF), granulocyte macrophage CSF (GM-CSF), macrophage CSF (M-CSF), interleukin-1 beta, interleukin-3, interleukin-6, interferon-gamma, tumor necrosis factor, lymphotoxin, leukemia inhibitory factor, and transforming growth factor-alpha.

Among the pathological conditions associated with an abnormal osteoclast development or function are conditions wherein increased bone resorption results in the development of fragile and/or brittle bone structure, such as osteoporosis, or increased bone absorption results in the development of excess bone mass, such as osteopetrosis. It is believed that the development of excess or deficient populations of osteoclasts or osteoblasts results from a corresponding lack or excess of specific cytokines in the blood.

Many of the known cytokines stimulate or inhibit blood cells. Several growth regulatory cytokines such as CSF-M, transforming growth factor alpha, interleukin-1 and tumor necrosis factor have been shown to stimulate marrow mononuclear cell proliferation. Although cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interleukin-6 (IL-6) may influence osteoclast formation and differentiation (Mundy, Trends in Endocrinol. & Metab. 1:307-311, 1990), these factors are not specific osteoclast growth regulatory factors.

REPORTED DEVELOPMENTS

Recently, Yoneda et al. isolated a human squamous cell tumor associated with leukocytosis and splenomegaly, hypercalcemia and increased osteoclastic bone resorption (Yoneda et al., J. Clin. Oncol. 9:468-477, 1991). Nude mice bearing this tumor also exhibited leukocytosis, splenomegaly, hypercalcemia and increased osteoclastic bone resorption (Yoneda et al., J. Clin. Oncol. 9:468-477, 1991; J. Clin. Invest. 87:977-985, 1991). When these tumors were surgically removed, there was a dramatic decrease in osteoclastic

bone resorption and leukocyte count. A biologically active polypeptide, osteoclastpoietic factor (OPF), was isolated from the conditioned media from cultured tumor cells (MH-85 cells). Among the biological activities of OPF was the ability to regulate the growth and/or differentiation of osteoclast cells (WO 93/01827 which is incorporated herein by reference).

The present invention relates to the isolation of an osteoclast stimulatory factor from an expression library derived from human marrow-derived osteoclastic cells and to the characterization of a polypeptide having biological activity including regulatory activity associated with the development of multinucleated bone cells.

SUMMARY OF THE INVENTION

The present invention relates to a biologically active polypeptide comprising the amino acid sequence shown in Figure 1 (SEQ ID NO: 2), or a biologically active sequence analogue thereof. Among the biological properties of the polypeptide of the present invention is the capability to regulate the growth and/or differentiation of osteoclast cells.

The invention further provides nucleotide sequences encoding said biologically active polypeptide. A preferred embodiment is the coding region (nucleotides 150-794) shown on Figure 1 (SEQ ID NO: 1).

In other embodiment, the invention provides monoclonal and polyclonal antibodies capable of specifically binding to the amino acid sequence of Figure 1, or to a biologically active sequence analogue thereof, as well as uses of these monoclonal and polyclonal antibodies therapeutically and diagnostically. The antibodies of the present invention are useful for affinity purification of the naturally occurring polypeptide as well as active fragments thereof, in assays for detecting the present polypeptide and for treating pathological conditions resulting from overproduction thereof. The assays provide a method for the clinical diagnosis and assessment of those diseases in which there is excess production of the naturally occurring polypeptide, and for monitoring treatment efficacy.

The invention also provides compositions, such as diagnostic and pharmaceutical compositions, containing the polypeptide of the present invention and methods of using these in treatment and diagnosis.

In another embodiment, the present invention provides a method for the treatment of bone diseases characterized by abnormal osteoclast activity such

as osteopetrosis, comprising administration of the present polypeptide to individuals in need of such treatment. Antagonists, such as the present antibodies to the present polypeptides, are useful for inhibiting bone resorption in a number of disease states where bone resorption is enhanced such as, but not limited to osteoporosis, Paget's disease, malignant diseases which affect the skeleton such as myeloma and breast cancer, and chronic inflammatory diseases which cause localized bone loss such as rheumatoid arthritis and periodontal disease. Treatment of these diseases may be accomplished by administration of antagonists such as neutralizing antibodies to this and related polypeptides to individuals in need of such treatment.

Other and further objects features and advantages will be apparent from the following description of the presently preferred embodiments of the invention, given for the purposes of disclosure when taken in conjunction with the accompanying drawing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cDNA sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of OSF.

Figure 2 shows a primary screening of the human osteoclast-like cell cDNA expression library. Conditioned media from 293 cells transiently transfected with pools (P1-P16) from the pcDNAI expression library were tested for the ability to enhance osteoclast-like MNC formation in human marrow cultures. Results shown are the mean ± S.E. for five replicate samples and were compared by the Student's t test. Results were considered significantly different for $p<0.05$ (*) or $p<0.01$ (**).

Figure 3 shows the ability of conditioned media from 5F cDNA-transfected 293 cells to enhance formation of MNCs in human bone marrow cultures. Results shown are the mean ± S.E. for five replicate samples and were compared by the Student's t test. Results were considered significantly different for $p<0.05$ (*).

Figure 4 shows the effect of recombinant OSF on the formation of MNCs in human bone marrow cultures.

Figure 5 shows the effect of 5F conditioned media on formation of MNCs in murine marrow cultures.

Figure 6 shows the effect of 5F conditioned media on mouse bone marrow pit formation.

Figure 7 shows the effect of recombinant OSF on fetal rat bone resorption.

DETAILED DESCRIPTION

The present invention relates to a biologically active polypeptide comprising the amino acid sequence shown in Figure 1 (SEQ ID NO: 2), or a biologically active sequence analogue thereof. The amino acid sequence of this polypeptide is distinct from the sequences of other proteins which have been shown to promote osteoclast formation.

"Biological activity" means one or more functions, effects of, activities performed or caused by a molecule in a biological context (that is, in an organism or in an in vitro facsimile). A characteristic biological activity of OSF is the ability to stimulate the growth and/or differentiation of osteoclast cells.

The term "biologically active polypeptide" means the naturally occurring polypeptide per se, as well as biologically active analogues thereof, synthetic produced polypeptides, natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The present invention encompasses OSF and biologically active fragments thereof, as well as biologically active sequence analogues thereof. Different alleles of OSF may exist in nature. These variations may be characterized by differences in the nucleotide sequence of the structural gene coding for proteins of identical biological function. The term "biologically active sequence analogue" includes analogues having single or multiple amino acid substitutions, deletions, additions, or replacements. All such allelic variations, modifications, and analogues resulting in derivatives of OSF which retain one or more of the biologically active properties of native OSF are included within the scope of this invention.

The present invention provides polypeptides in substantially homogeneous form. The term "substantially homogeneous" means that the polypeptide is essentially free of other proteins normally associated with the polypeptide in its natural state. The term "substantially homogeneous" is not meant to exclude artificial or synthetic mixtures of the polypeptide with other compounds.

A "substantially homogeneous" nucleic acid is a nucleic acid essentially free of other nucleic acids normally associated with the nucleic acid in its

natural state. The term "substantially homogeneous" is not meant to exclude artificial or synthetic mixtures of the nucleic acid with other compounds.

The present invention also provides methods for purifying OSF. In one embodiment, the present invention provides a method for purifying recombinant OSF, which is produced by transforming E. coli with a vector comprising the OSF cDNA.

In another embodiment, the present invention provides a method for purifying OSF comprising contacting a medium containing OSF mixed with other proteins with an antibody which binds to at least one epitope of the OSF molecule, removing the antibody-OSF complex, releasing the OSF from the antibody and separating the OSF from the antibody. In a preferred embodiment the antibody is bound to a solid support. The choice of solid support and methods for binding the antibody to the solid support are well known to those skilled in the art.

The present invention also provides nucleic acids encoding the biologically active polypeptide of the present invention. A preferred embodiment is the coding region (nucleotides 150-794) shown on Figure 1 (SEQ ID NO: 1).

The biologically active polypeptides of the present invention may be prepared utilizing recombinant technology. A recombinant DNA molecule coding for any of the polypeptides of the present invention can be used to transform a host using techniques known to those of ordinary skill in the art.

General methods for the cloning and expression of recombinant molecules are described in Maniatis (Molecular Cloning, Cold Spring Harbor Laboratories, 1982), and in Ausubel (Current Protocols in Molecular Biology, Wiley and Sons, 1987), which are incorporated herein by reference.

A "plasmid" is a non chromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a prokaryotic or eukaryotic host cell, the characteristics of that cell may be changed (or transformed) as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (TetR) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

A "vector" is a plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, typically characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in

a determinable fashion for the insertion of heterologous DNA without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of expression control regions such as promoters or binding sites, and which may contain a selectable gene marker suitable for use in the identification of host cells transformed therewith, e.g., tetracycline resistance or ampicillin resistance.

An "expression vector" is a vector which is capable of expressing a peptide encoded by heterologous DNA sequences contained in the vector, such as the cDNA encoding OSF. The heterologous DNA sequences are operably linked to regulatory sequences which are capable of regulating expression of the peptide.

An expression vector typically contains an origin of replication, promoter(s), terminator(s), a ribosome binding site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. These expression vectors must be replicable in the host organisms or systems either as episomes, bacteriophage, or as an integral part of the chromosomal DNA. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

Prokaryotic cells are preferred for the cloning of DNA sequences and in the construction of vectors. Cell lines derived from multicellular organisms may also be used as hosts. Examples of such hosts are the VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI38, BHK, COS-7, and MDCK cell lines. Expression vectors for such cells ordinarily include an origin of replication, a promoter located in front of the gene to be expressed, RNA splice sites (if necessary), and transcriptional termination sequences.

For use in mammalian cells, the control functions (promoters and enhancers) on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently, Simian Virus 40 (SV40). Eukaryotic promoters, such as the promoter of the murine metallothionein gene [Paulakis and Hamer, Proc. Natl. Acad. Sci. 80:397-401 (1983)], may also be used. Further, it is also possible, and often desirable, to utilize the promoter or control sequences which are naturally associated with desired gene sequence, provided such control sequences are compatible with the host system. To increase the rate of transcription, eukaryotic enhancer sequences can be obtained from a variety of animal cells or oncogenic retroviruses such as the mouse sarcoma virus.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as that provided by SV40 or other viral sources, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The present invention includes any host modified according to the methods described, or modified by any other methods commonly known to those of ordinary skill in the art, which yields a prokaryote or eukaryote expressing the gene for OSF.

Host cells used to prepare the polypeptides of the present invention can be of a variety of chemical compositions. The polypeptide may be produced having methionine as its first amino acid. This methionine is present by virtue of the ATG start codon naturally existing at the origin of the structural gene or by being engineered before a segment of the structural gene. The protein may also be intracellularly or extracellularly cleaved, giving rise to the amino acid which is found naturally at the amino terminus of the polypeptide.

Recombinant host cells are cells which have been transformed with vectors constructed using recombinant DNA techniques. The polypeptides of the present invention or fragments thereof produced by such cells are referred to as "recombinant polypeptides of the present invention."

By providing the DNA sequences, and recombinant DNA molecules, the present invention also provides probes and methods to identify cells containing or lacking these sequences, and means to administer these sequences to cells. This will enable the establishment of systems in which the recombinant protein is produced after transfection of an expression vector into appropriate host cells. Additionally, the present invention provides a means to inhibit the expression of the novel sequences by providing an antisense RNA sequence which, when administered to a cell, or when the DNA encoding said antisense RNA is administered to a cell, said DNA sequence will produce an antisense RNA which can bind to and therefore block the expression of the RNA encoding the novel polypeptides of the present invention. It will also be apparent to one of skill in the art from this disclosure that antibodies against any of the proteins of the present invention can be utilized to block the binding of ligands to the polypeptides and to target drugs or other agents (such as labels) to the cells expressing these polypeptides.

Monoclonal antibodies of the present invention may be prepared using the method of Mishell, B. B., et al., Selected Methods In Cellular Immunology,

(W.H. Freeman, ed.) San Francisco (1980). The biologically active polypeptide of the present invention is used as the antigen for the production of these antibodies. Briefly, OSF peptide is used to immunize spleen cells of Balb/C mice. The immunized spleen cells are fused with FO myeloma cells. Fused cells containing spleen and myeloma cell characteristics are isolated by growth in HAT medium, a medium which kills both parental cells, but allows the fused products to survive and grow.

The anti-OSF antibodies are useful in the treatment of disease states caused by increased levels of OSF in the individual. Neutralizing antibodies can inhibit the activity of the excessively produced OSF in these individuals. These treatments include administration of anti-OSF monoclonal antibodies to individuals suffering from osteoporosis, malignant diseases which affect the skeleton such as myeloma and breast cancer, and chronic inflammatory diseases which cause localized bone loss such as rheumatoid arthritis and periodontal disease.

The anti-OSF antibodies are also useful in assays for detecting or quantitating levels of OSF. These assays provide a clinical diagnosis and assessment of those diseases in which excess production of these factors occurs, and a method for monitoring treatment efficacy.

Synthetic antagonists to OSF have the same beneficial therapeutic effect as neutralizing antibodies in those diseases characterized by the overproduction of OSF.

The term "individual" is meant to include any animal, preferably a mammal, and most preferably a rodent, cat, dog, cow or human.

The techniques for detectably labeling the homogeneous OSF and the monoclonal antibodies thereto of the present invention with a radiolabel, an enzyme label, or a fluorescent label are well known to those of skill in the art. Reference can be made to Chard, An Introduction To Radioimmunoassay And Related Techniques, North-Holland Publishing Co., Amsterdam-NY-Oxford (1978), The Enzyme-Linked Immunoadsorbent Assay (ELISA) by Voller, A., et al., Dynatech Europe Borough House, Rue du Pre, Guernsey, Great Britain, and Radioiodination Techniques, Review 18, Amersham Corporation, by A. E. Bolton, all incorporated herein by reference. Preferably, the purified OSF is labeled with ^{125}I using the Bolton/Hunter reagent which involves succinylation of the free N-terminals and lysine. DNA probes may also be labeled with a detectable label. Commonly used detectable labels are radioactive labels including, but not limited to, ^{32}P, ^{14}C, ^{125}I, ^{3}H and ^{35}S. Biotin labeled

nucleotides can be incorporated into DNA or RNA by nick translation, enzymatic, or chemical means. The biotinylated probes are detected after hybridization using avidin/streptavidin, fluorescent, enzymatic or colloidal gold conjugates. Nucleic acids may also be labeled with other fluorescent compounds, with immunodetectable fluorescent derivatives or with biotin analogues. Nucleic acids may also be labeled by means of attaching a protein. Nucleic acids cross-linked to radioactive or fluorescent histone HI, enzymes (alkaline phosphatase and peroxidases), or single-stranded binding (ssB) protein may also be used.

Administration of the compounds useful in the method of present invention may be by parenteral, intravenous, intramuscular, subcutaneous, rectal or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the pathological state being treated. The effective compound useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the compounds used in the method of the present invention have suitable solubility properties for use in the method of the present invention.

As used herein the term "salts" refers to both salts of carboxy groups of the polypeptide or protein chain and to acid addition salts of amino groups of the polypeptide chain. Salts of the carboxy group may be formed with either inorganic or organic bases by means known in the art per se. Inorganic salts include, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like. Salts with organic bases include those formed, for example, with amines such as triethanolamine, arginine, lysine, piperidine, caffeine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

Both the salts and the derivatives encompassed by the invention include those which are therapeutically or diagnostically acceptable, i.e., those which do not destroy the biologic, immunogenic, or binding activity of OSF depending on the functional activity desired to be utilized.

Having now generally described the invention, a more complete understanding can be obtained by reference to the following specific

examples. These examples are provided for the purpose of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

Bone marrow multinucleated cell culture

Human long term marrow cultures were performed as described previously (Takahashi et al., J. Clin. Invest. 83:543-550, 1989; MacDonald et al., Endocrinology 120:2326-2333, 1987). In brief, human bone marrow nonadherent mononuclear cells from normal volunteers were cultured at 10^6 cells/ml in α-MEM, 20% horse serum with or without 10^{-8}M 1,25-$(OH)_2D_3$. Half of the medium was changed weekly. After 3 weeks, the cultures were harvested and stained with the 23c6 monoclonal antibody or lysed for the TRAP assay.

TRAP assay

Human long term marrow cultures were prepared as described above, and after 3 weeks, the cells were washed twice with phosphate-buffered saline and treated with trypsin-EDTA for 10 min to remove the lightly adherent mononuclear cells. The remaining adherent cells were then washed with phosphate-buffered saline vigorously four times, and fresh medium was added to each culture. The cells were cultured overnight and lysed in 0.2 ml of Triton X-100 (0.05%, v/v). Aliquots of the lysate (40 µl) were incubated with 160 µl of 2 mM methylumbeliferyl phosphate, pH 5.0, in 0.48 M acetate buffer (0.48 M sodium acetate, 0.48 M acetic acid, pH 5.0), and 20 mM tartaric acid. Samples were incubated for 30 min at 37°C, and the reaction was terminated with 100 µl of stop solution containing 50 mM glycine, 50 mM EDTA, pH 10.4. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 448 nm using a fluorometer (Fluoroskan, Flow Instruments, McLean, VA) as described previously (Oreffo et al., J. Bone Min. Res. 3:203-210, 1988). In addition, normal bone marrow cells were cultured for 3 weeks with medium only, 1,25-$(OH)_2D_3$ (10^{-8}M), or IL-1β (10 ng/ml), and the numbers of $23c6^+$ MNC and TRAP activities in the cell lysate from simultaneous cultures were compared. The amount of TRAP activity in the

lysate was highly correlated (r=0.9) with the number of $23c6^+$ MNC present in the culture.

EXAMPLE 2

Construction of a mammalian cDNA expression library from highly purified human osteoclast-like multinucleated cells.

Human bone marrow-derived nonadherent mononuclear cells from 20 separate normal marrow donors were cultured at 10^6 cells/ml in the presence of 10^{-8}M 1,25-$(OH)_2D_3$ in T-75 culture flasks for 3 weeks as described previously (Takahashi et al., J. Clin. Invest. 83:543-550, 1989). The cells were harvested from the plastic culture flasks by treatment of the cultures with chymopapain (300 units/ml) for 30 min at 37°C, and the released cells were pelleted by centrifugation at 1500 rpm for 5 min. The cells were resuspended in serum-free α-minimum essential medium (α-MEM), and the osteoclast-like multinucleated cells (MNC) were purified by immune panning with the 23c6 monoclonal antibody (Ohsaki et al., Endocrinology 131: 2229-2234, 1992). The 23c6 monoclonal antibody was generously provided by Dr. Michael Horton (St. Bartholomew's Hospital, London, UK). We have previously shown that MNC that react with the 23c6 antibody are the osteoclast-like cells in these cultures (Kurihara et al., Endocrinology 126: 2733-2741, 1990). $Poly(A)^+$ RNA was isolated from the $23c6^+$ cell fraction using oligo(dT)-cellulose spin columns. A cDNA library was made in the λgt11 vector using a cDNA synthesis kit (Pharmacia) following the manufacturer's protocol. The cDNA inserts were excised from the λgt11 vectors by digestion at 37°C for 2 h with 30 units of EcoRI. The cDNAs were then size-fractionated, and the 0.5-8 kilobase cDNAs were cloned into the pcDNAI mammalian expression vector (Invitrogen, San Diego, CA).

The pcDNAI expression vector contains enhancer-promoter sequences from the immediate early gene of the human cytomegalovirus (CMV), which can be transactivated by the adenovirus EIA protein. This results in high levels of gene expression when genes are transiently transfected into mammalian cells such as human 293 cells. The pcDNAI vector also contains a Col E1 origin for growth in E. coli, and a gene encoding the tRNA suppressor F gene (sup F). The pcDNAI vector is efficiently replicated when transformed into a strain of E. coli such as MC1061/P3, which harbors the plasmid P3. P3 is a

low-copy 60 kb episome which encodes a kanamycin resistance gene as well as amber mutants of the tetracycline and ampicillin resistance genes. When E. coli carrying the P3 plasmid such as MC1061/P3 are transformed with sup F vectors such as pcDNAI, they are rendered both tetracycline and ampicillin resistant by suppression of the amber mutations.

The cDNAs in pcDNAI were transformed into MC1061/P_3 Escherichia coli, and transformants were selected with tetracycline (10 μg/ml) and ampicillin (40 μg/ml). The resulting pcDNAI expression library contained $4x10^5$ cDNA clones. This library was divided into 200 pools containing 2000 clones each. Replicate plate lifts of each pool were performed using nitrocellulose filters, and the plates were stored at -20°C as described (Hanahan and Meselson, Gene 10: 63-67, 1980).

EXAMPLE 3

Screening of the mammalian cDNA expression library.

The cDNA expression library was screened by testing the effects of conditioned media from transfected mammalian 293 cells for the capacity to enhance osteoclast-like MNC formation in human marrow cultures.

Cesium chloride gradient purified plasmid DNA (10 μg) from each of the 200 expression library pools was transiently transfected into 293 cells grown in individual 35-mm wells ($4x10^5$ cells/well) using a calcium phosphate method (using a kit from Stratagene according to the manufacturer's protocol). Twelve hours after the start of the DNA transfection process, the cells were fed with 1.5 ml of serum-free Dulbecco's modified Eagle's medium. Conditioned media from each pool were collected after 48 h and tested at different concentrations (0.1-10%, v/v) for the capacity to enhance MNC formation in human marrow cultures. Conditioned media were added to normal human bone marrow long term culture in the presence or absence of 10^{-8}M 1,25-$(OH)_2D_3$. At the end of the three week culture period, the cells were stained for 23c6 reactivity, or the MNC were lysed for determining tartrate-resistant acid phosphatase (TRAP) activity as described above. Pools that increased TRAP activity were retested to confirm that they enhanced osteoclast-like MNC formation by counting the number of 23c6 antibody-reactive MNC formed in the marrow cultures. The number of $23c6^+$ MNC were counted and compared with cultures not containing conditioned medium or with cultures treated with conditioned

medium from 293 cells that had not been transfected. A typical screening experiment is shown in Figure 2. In this experiment, 23c6+ MNC formation was increased more than 2-fold by conditioned media from seven of the pools tested.

A total of nine pools that stimulated MNC formation in cultures treated with 1,25-$(OH)_2D_3$ were detected among the original 200 pools. The positive pools were screened for known factors that enhance osteoclast formation (IL-1, IL-6, granulocyte macrophage colony stimulating factor, macrophage colony stimulating factor, tissue necrosis factor, and parathyroid hormone) by reverse transcription-PCR. IL-1ß and IL-6 cDNAs were detected in three of the nine positive pools, and these three pools were not screened further.

The remaining six positive cDNA pools were separated into 12 subpools each, containing 100-200 clones/subpool. The individual pools were transfected into 293 cells, and the conditioned media were tested for their effects on MNC formation. Because of the large number of cultures, the initial assay was for TRAP activity in cell lysates from the human bone marrow cultures. Because there are many TRAP+ mononuclear cells in human bone marrow cultures, the cultures were first treated with trypsin-EDTA to remove the majority of the mononuclear cells. Greater than 90% of the MNC were not removed, and few (<5%) of the mononuclear cells remained. After an overnight incubation with fresh medium, the remaining cells were lysed, and the TRAP activity in the cell lysate was determined. When this TRAP assay was used to screen the previous six positive pools, TRAP activity was significantly increased in cultures treated with conditioned media from six positive pools compared to cultures treated with conditioned media from nontransfected 293 cells or cultures not treated with conditioned media. One of the positive pools (no. 12) was further subdivided into several subpools and screened by the TRAP assay. Each positive subpool, containing 100-200 clones, was separated into 96 or 192 subpools that had one clone per pool. Conditioned medium from each of these clones was treated for its capacity to increase MNC formation.

The first positive clone identified was 5F. The effects of conditioned media from 5F cDNA-transfected 293 cells was tested on osteoclast-like MNC formation in mouse (see Example 5, below) and in human bone marrow cultures.

The 5F conditioned medium increased MNC formation in human bone marrow cultures, as shown in Figure 3. An increase in MNC formation was

seen at concentrations as low as 0.05% (v/v), in the absence or presence of $1,25\text{-}(OH)_2D_3$

The 5F cDNA was sequenced using a dideoxyoligonucleotide chain termination method (using a kit from USB according to the manufacturer's directions). The 5F cDNA encoded a 23kd peptide. The nucleotide sequence and deduced amino acid sequence are shown in Figure 1. The sequence was compared to sequences in GenBank and was found to be unique, with no other sequence having complete homology.

EXAMPLE 4

Expression of OSF in E. coli

The 5F cDNA was introduced into a pET vector (Novagen Inc., Madison, WI), and the 5F clone was expressed in the BL21(DE3)pLysS strain of E. coli.

The pET vector is designed for expression of target cDNA inserts as stable fusion proteins under the control of the bacteriophage T7 promoter. The E. coli host BL21(DE3)pLysS contains a chromosomal copy of the T7 RNA polymerase gene under LAC UV5 control. Expression vectors containing a target cDNA in pET are transformed into BL21(DE3)pLysS, and cDNA expression is then induced by the addition of IPTG. The pET vector has the advantage of placing a six histidine tag sequence at the N-terminal end of the recombinant protein, which allows easy purification of the recombinant protein. The histidine tag can then be removed.

The 5F cDNA was cloned into the pET vector, resulting in the expression vector 5FpET2. The 5FpET2 expression vector was transformed into the BL21(DE3)pLysS strain of E. coli using a calcium phosphate method. Expression was induced by IPTG. The recombinant OSF protein was affinity purified using a metal chelation resin containing immobilized divalent Nickel cations (His.Bind Resin, Novagen Inc.), which efficiently binds the histidine tag sequence. After washing away unbound proteins, the recombinant OSF fusion protein was eluted from the resin with 50 mM sodium phosphate buffer, pH 8.0 containing 0.3 M NaCl and 250 mM imidazole. The histidine tag sequence was then removed by thrombin cleavage, resulting in recombinant OSF.

The recombinant OSF induced osteoclast formation in both human and murine marrow cultures (Figure 4).

EXAMPLE 5

The effect of 5F conditioned media on Murine bone marrow cultures

Bone marrow from CB57 black mouse tibiae that had been aseptically removed was obtained by flushing the tibiae with 1 ml of α-MEM using a tuberculin syringe fitted with a 25-gauge needle. The bone marrow-derived cells were washed twice and resuspended in α-MEM, 20% fetal calf serum, and the cells depleted of cells adherent to plastic by incubating the marrow cell suspension in sterile 10-cm tissue culture dishes for 2 h. The nonadherent marrow cells were collected and cultured for 6 days in 24-well plates at a density of 1.0×10^6 cells/well in α-MEM, 10% fetal calf serum supplemented with 1,25-$(OH)_2D_3$ (10^{-8} or 10^{-9}M) as described by Takahashi et al., Endocrinology 122:1373-1382, 1988). The cultures were fixed with 4.5 mM citric acid, 2.25 mM sodium citrate, 3 mM sodium chloride, 3% formaldehyde, and acetone and were washed twice in distilled water. The cultures were then stained for TRAP using an acid phosphatase staining kit (Sigma) and counterstained with methyl green. The TRAP-positive multinucleated cells containing three or more nuclei were then scored.

In mouse bone marrow cultures, the 5F conditioned media increased $TRAP^+$ MNC formation two-fold at concentrations of 0.1-5% (v/v) in the presence of 10^{-8} or 10^{-9}M of vitamin D_3 (Figure 5). The 5F conditioned medium also enhanced $TRAP^+$ MNC formation in mouse bone marrow cultures in the absence of 1,25-$(OH)_2D_3$.

EXAMPLE 6

The effect of 5F conditioned media and recombinant OSF on dentine resorption by isolated osteoclasts

Selected murine marrow cultures were overlaid with dentine at the start of the cultures. After 6 days, the dentine slices were removed and stained for TRAP. The number of $TRAP^+$ MNC on each dentine slice was counted manually, and the dentine slices were washed in distilled water. The area of the slice resorbed was determined in a blinded fashion with an inverted microscope using bright field reflected microscopy at 50x magnification with a Bioquant image analysis system (R and M Biometrics, Nashville, TN). The area

resorbed was determined with Java image analysis software (Jandel Scientific, Corte Madona, CA).

In separate murine marrow cultures which were treated with 5F conditioned media, the effects on the capacity of the osteoclasts to cause resorption pits on slices of sperm whale dentine were examined. The recombinant OSF enhanced by two to three-fold the capacity of isolated osteoclasts to cause bone resorption (Figure 6).

EXAMPLE 7

Effects of recombinant OSF on bone resorption in fetal rat organ cultures in vitro

Timed-pregnant rats were injected with 250 µCi of $^{45}CaCl_2$ at day 18 of gestation, and 1 day later the rats were killed by cervical dislocation, and the embryos were removed. The explanted radii and ulnae were cultured on circles of membrane filter, mixed ester, 0.45 µm (Whatman, Hillsboro, OR) on stainless steel grids in 0.5 ml of chemically defined medium (Sigma) supplemented with 1 mg/ml bovine serum albumin (Sigma) and penicillin-streptomycin (50 units/ml and 50 mg/ml), in a controlled atmosphere of 5% CO_2 in air at 37°C, as modified from Raisz and Niemann (Endocrinology 85: 446-452, 1969). The radii and ulnae were incubated for 24 h in control medium to allow for the removal of the exchangeable ^{45}Ca before transferring to equilibrated control or experimental medium. Experimental media contained either 10^{-9} M 1,25-$(OH)_2D_3$, recombinant OSF (2 µg/ml), or a combination of OSF and 1,25-$(OH)_2D_3$. Control or experimental media were then changed after 72 h, with bone explants incubated for a total of 5 days. Bone-resorbing activity was measured as percentage of total ^{45}Ca released from the bone into the medium over 5 days of incubation.

The recombinant molecule enhanced two-fold the ^{45}Ca release from organ cultures of fetal rat long bones in the presence of 1,25-$(OH)_2D_3$ (10^{-9}M) (Figure 7).

EXAMPLE 8

Expression of OSF

RT-PCR analysis of a variety of tissues demonstrated the expression of OSF in peripheral blood mononuclear cells and highly purified osteoclasts derived from human giant cell tumors of bone and pagetic bone.

EXAMPLE 9

Preparation of Monoclonal Antibodies to OSF

Monoclonal antibodies (MAb) to OPF are made using the in vitro immunization technique of Van Ness et al., (Nature 301: 425-427, 1983). Spleen cells of 8 to 12 week-old female Balb/C mice are immunized with 100 ng recombinant OSF in the presence of 100 µg N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP, Sigma), 125 µg lipopolysaccharide (LPS, Difco) and 500 µl culture supernatants of concanavalin A (50 µg/ml)-stimulated spleen cells (5 x 10^6/ml) in 5 ml IMDM supplemented with 20% FBS in 6-well plates for 4 days. The immunized spleen cells are then hybridized with mouse myeloma FO cells (kindly provided by Dr. Eguchi, Kaneka, Japan) at a ratio of 2:1 in the presence of 50% polyethylene glycol (1500, Boehringer-Manheim). After the hybridization, the cells are suspended in 120 ml IMDM supplemented with 10% FBS and 0.5 ml cell suspensions are inoculated onto each well in 48-well plates. After 24 hours, 5 x 10^5/well thymocytes in 0.5 ml HAT medium are plated onto each well as a feeder layer. The plates are cultured for 14 days in HAT medium (Flow) and then for 7 to 10 days in HT medium (Flow). The cells are fed fresh medium every 2 days. The culture supernatants harvested from the wells in which growing hybridoma cells cover 50% of the surface area are screened for their cross reactivity with OSF by enzyme-linked immunosorbent assay (ELISA).

EXAMPLE 10

Preparation of Polyclonal Antibodies to OSF

Rabbits (male, 6 to 8 week-old) are subcutaneously injected (10 sites/animal, 100 µl/site) with 1 µg recombinant OSF in complete Freund's adjuvant. Four weeks after the first immunization, animals are boosted intramuscularly with 1 µg recombinant OSF in incomplete Freund's adjuvant. The animals are boosted three times. At the time of each each booster, blood is drawn from an ear vein and tested for its reactivity against OSF by Western blot or by ELISA.

DEPOSIT OF STRAINS USEFUL IN PRACTICING THE INVENTION

Deposits of biologically pure cultures of the following strains were made under the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, The accession numbers indicated were assigned after successful viability testing, and the requisite fees were paid.

Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner of the United States Patent and Trademark Office to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122, or if and when such access is required by the Budapest Treaty. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application and said cultures will remain permanently available for a term of at least five years after the most recent request for the furnishing of samples and in any case for a period of at least 30 years after the date of the deposits. Should the cultures become nonviable or be inadvertantly destroyed, they will be replaced with viable cultures(s) of the same taxonomic description.

Strain/Plasmid	ATCC No.	Deposit Date
5FpET2 plasmid		

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The peptides, antibodies,

methods, procedures and techniques described herein are presented as representative of the preferred embodiments, or intended to be exemplary and not intended as limitations on the scope of the present invention. Changes therein and other uses will occur to those of skill in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: OSTEOSA INC.

(ii) TITLE OF INVENTION: Osteoclast Growth Regulatory Factor

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Campbell & Flores LLP
(B) STREET: 4370 La Jolla Village Drive, Suite 700
(C) CITY: La Jolla
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92122

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Paul C. Steinhardt
(B) REGISTRATION NUMBER: 30,806
(C) REFERENCE/DOCKET NUMBER: FP-ND 2122

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 535-9001
(B) TELEFAX: (619) 535-8949

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 901 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 150..794

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAGAACTGG GGTGCCCGGA GTGCCAGGTG GCGGGCAAGC GGTGGGCTTT TCGGCGGGGT 120

```
CTTTAGGATT TGCAGCTCCA GGAAGCGAG ATG TCG AAG CCG CCA CCC AAA CCA        173
                                Met Ser Lys Pro Pro Pro Lys Pro
                                  1               5

GTC AAA CCA GGG CAA GTT AAA GTC TTC AGA GCC CTG TAT ACG TTT GAA        221
Val Lys Pro Gly Gln Val Lys Val Phe Arg Ala Leu Tyr Thr Phe Glu
     10                  15                  20

CCC AGA ACT CCA GAT GAA TTA TAC TTT GAG GAA GGT GAT ATT ATC TAC        269
Pro Arg Thr Pro Asp Glu Leu Tyr Phe Glu Glu Gly Asp Ile Ile Tyr
 25                  30                  35                  40

ATT ACT GAC ATG AGC GAT ACC AAT TGG TGG AAA GGC ACC TCC AAA GGC        317
Ile Thr Asp Met Ser Asp Thr Asn Trp Trp Lys Gly Thr Ser Lys Gly
                 45                  50                  55

AGG ACT GGA CTA ATT CCA AGC AAC TAT GTG GCT GAG CAG GCA GAA TCC        365
Arg Thr Gly Leu Ile Pro Ser Asn Tyr Val Ala Glu Gln Ala Glu Ser
            60                  65                  70

ATT GAC AAT CCA TTG CAT GAA GCA GCA AAA AGA GGC AAC TTG AGC TGG        413
Ile Asp Asn Pro Leu His Glu Ala Ala Lys Arg Gly Asn Leu Ser Trp
        75                  80                  85

TTG AGA GAG TGT TTG GAC AAC AGA GTG GGT GTT AAT GGC TTA GAC AAA        461
Leu Arg Glu Cys Leu Asp Asn Arg Val Gly Val Asn Gly Leu Asp Lys
     90                  95                 100

GCT GGA AGC ACT GCC TTA TAC TGG GCT TGC CAC GGG GGC CAC AAA GAT        509
Ala Gly Ser Thr Ala Leu Tyr Trp Ala Cys His Gly Gly His Lys Asp
105                 110                 115                 120

ATA GTG GAA ATG CTA TTT ACT CAA CCA AAT ATT GAA CTG AAC CAG CAG        557
Ile Val Glu Met Leu Phe Thr Gln Pro Asn Ile Glu Leu Asn Gln Gln
                125                 130                 135

AAC AAG TTG GGA GAT ACA GCT TTC GAT GCT GCT GCC TGG AAG GGT TAT        605
Asn Lys Leu Gly Asp Thr Ala Phe Asp Ala Ala Ala Trp Lys Gly Tyr
            140                 145                 150

GCA GAT ATC GTC CAG TTG CTT CTG GCA AAA GGT GCT AGA ACA GAC TTA        653
Ala Asp Ile Val Gln Leu Leu Leu Ala Lys Gly Ala Arg Thr Asp Leu
        155                 160                 165

AGA AAC ATT GAG AAG AAG CTG GCC TTC GAC ATG GCT ACC AAT GCT GCC        701
Arg Asn Ile Glu Lys Lys Leu Ala Phe Asp Met Ala Thr Asn Ala Ala
    170                 175                 180

TGT GCA TCT CTC CTG AAA AAG AAA CAG GGA ACA GAT GCA GTT CGA ACA        749
Cys Ala Ser Leu Leu Lys Lys Lys Gln Gly Thr Asp Ala Val Arg Thr
185                 190                 195                 200

TTA AGC AAT GCC GAG GAC TAT CTC GAT GAT GAA GAC TCA GAT TAA            794
Leu Ser Asn Ala Glu Asp Tyr Leu Asp Asp Glu Asp Ser Asp  *
                205                 210                 215

TTCCTTTCTG GAGCTTTGAG ATCTAAAACT TCTGTTGCTT TTGCCATTCC AAAACTTTGT      854

CTTTGCCAGA AAAGTGTTGG TAACTATAAA GAAAATATAT ATGAAAA                    901
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Lys Pro Pro Pro Lys Pro Val Lys Pro Gly Gln Val Lys Val
 1               5                  10                  15

Phe Arg Ala Leu Tyr Thr Phe Glu Pro Arg Thr Pro Asp Glu Leu Tyr
            20                  25                  30

Phe Glu Glu Gly Asp Ile Ile Tyr Ile Thr Asp Met Ser Asp Thr Asn
        35                  40                  45

Trp Trp Lys Gly Thr Ser Lys Gly Arg Thr Gly Leu Ile Pro Ser Asn
    50                  55                  60

Tyr Val Ala Glu Gln Ala Glu Ser Ile Asp Asn Pro Leu His Glu Ala
65                  70                  75                  80

Ala Lys Arg Gly Asn Leu Ser Trp Leu Arg Glu Cys Leu Asp Asn Arg
                85                  90                  95

Val Gly Val Asn Gly Leu Asp Lys Ala Gly Ser Thr Ala Leu Tyr Trp
            100                 105                 110

Ala Cys His Gly Gly His Lys Asp Ile Val Glu Met Leu Phe Thr Gln
        115                 120                 125

Pro Asn Ile Glu Leu Asn Gln Gln Asn Lys Leu Gly Asp Thr Ala Phe
    130                 135                 140

Asp Ala Ala Ala Trp Lys Gly Tyr Ala Asp Ile Val Gln Leu Leu Leu
145                 150                 155                 160

Ala Lys Gly Ala Arg Thr Asp Leu Arg Asn Ile Glu Lys Lys Leu Ala
                165                 170                 175

Phe Asp Met Ala Thr Asn Ala Ala Cys Ala Ser Leu Leu Lys Lys Lys
            180                 185                 190

Gln Gly Thr Asp Ala Val Arg Thr Leu Ser Asn Ala Glu Asp Tyr Leu
        195                 200                 205

Asp Asp Glu Asp Ser Asp  *
    210                 215
```

What is claimed as new and intended to be covered by letters patent of the United States is:

1. A substantially homogeneous polypeptide comprising the amino acid sequence shown on Figure 1 (SEQ ID NO: 2), or a biologically active fragment or sequence analogue thereof.

2. The polypeptide of claim 1 wherein said polypeptide has a molecular weight of less than or equal to about 25,000 daltons.

3. The polypeptide of claim 1 wherein said polypeptide is capable of regulating the growth and/or differentiation of osteoclast cells.

4. An isolated DNA molecule comprising a DNA sequence selected from the group consisting of:

(a) nucleotides 150-791 of the sequence shown on Figure 1 (SEQ ID NO: 1), or its complementary strand;

(b) DNA sequences which hybridize to the sequences in (a); and

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the sequences in (a) or (b).

5. The DNA of claim 4, which encodes the peptide of Figure 1 (SEQ ID. NO: 2).

6. The DNA of claim 4, in which the DNA sequence is operably linked to regulatory control sequences.

7. A plasmid comprising the DNA of claim 6.

8. An expression vector comprising the DNA of claim 6.

9. A recombinant cell transformed with the plasmid of claim 7.

10. A recombinant cell transformed with the expression vector of claim 8.

11. The DNA of claim 4, comprising a DNA sequence consisting of nucleotides 150-791 on Figure 1 (SEQ ID NO: 1).

12. A nucleic acid probe comprising at least 20 sequential nucleotides of the DNA of claim 4.

13. An antibody capable of specifically binding to the polypeptide of claim 1.

14. The antibody of claim 13 wherein said antibody is a monoclonal antibody.

15. The antibody of claim 13 wherein said antibody is a polyclonal antibody.

16. A hybridoma cell line capable of producing the antibody of claim 14.

17. A pharmaceutical composition comprising the polypeptide of claim 1 and a pharmaceutically acceptable carrier.

18. A pharmaceutical composition comprising the antibody of claim 13 and a pharmaceutically acceptable carrier.

19. A method of treating osteopetrosis in an individual which comprises administering an effective amount of the pharmaceutical composition of claim 17 to an individual in need of said treatment.

20. A method of treating osteoporosis in an individual which comprises administering an effective amount of the pharmaceutical composition of claim 18 to an individual in need of said treatment.

```
CTCTTCCCGCAGCCAAGGGTGGGCGCCGGTCCTAGGAGGCGACGGTTGTAAGCCAGACAA      60

AAAGAACTGGGGTGCCCGGAGTGCCAGGTGGCGGGCAAGCGGTGGGCTTTTCGGCGGGGT     120

CTTTAGGATTTGCAGCTCCAGGAAGCGAGATGTCGAAGCCGCCACCCAAACCAGTCAAAC     180
                                M  S  K  P  P  P  K  P  V  K  P

CAGGGCAAGTTAAAGTCTTCAGAGCCCTGTATACGTTTGAACCCAGAACTCCAGATGAAT     240
  G  Q  V  K  V  F  R  A  L  Y  T  F  E  P  R  T  P  D  E  L

TATACTTTGAGGAAGGTGATATTATCTACATTACTGACATGAGCGATACCAATTGGTGGA     300
  Y  F  E  E  G  D  I  I  Y  I  T  D  M  S  D  T  N  W  W  K

AAGGCACCTCCAAAGGCAGGACTGGACTAATTCCAAGCAACTATGTGGCTGAGCAGGCAG     360
  G  T  S  K  G  R  T  G  L  I  P  S  N  Y  V  A  E  Q  A  E

AATCCATTGACAATCCATTGCATGAAGCAGCAAAAAGAGGCAACTTGAGCTGGTTGAGAG     420
  S  I  D  N  P  L  H  E  A  A  K  R  G  N  L  S  W  L  R  E
```

FIG. IA

```
AGTGTTTGGACAACAGAGTGGGTGTTAATGGCTTAGACAAAGCTGGAAGCACTGCCTTAT      480
  C  L  D  N  R  V  G  V  N  G  L  D  K  A  G  S  T  A  L  Y

ACTGGGCTTGCCACGGGGGCCACAAAGATATAGTGGAAATGCTATTTACTCAACCAAATA      540
  W  A  C  H  G  G  H  K  D  I  V  E  M  L  F  T  Q  P  N  I

TTGAACTGAACCAGCAGAACAAGTTGGGAGATACAGCTTTCGATGCTGCTGCCTGGAAGG      600
  E  L  N  Q  Q  N  K  L  G  D  T  A  F  D  A  A  A  W  K  G

GTTATGCAGATATCGTCCAGTTGCTTCTGGCAAAAGGTGCTAGAACAGACTTAAGAAACA      660
  Y  A  D  I  V  Q  L  L  L  A  K  G  A  R  T  D  L  R  N  I

TTGAGAAGAAGCTGGCCTTCGACATGGCTACCAATGCTGCCTGTGCATCTCTCCTGAAAA      720
  E  K  K  L  A  F  D  M  A  T  N  A  A  C  A  S  L  L  K  K

AGAAACAGGGAACAGATGCAGTTCGAACATTAAGCAATGCCGAGGACTATCTCGATGATG      780
  K  Q  G  T  D  A  V  R  T  L  S  N  A  E  D  Y  L  D  D  E

AAGACTCAGATTAATTCCTTTCTGGAGCTTTGAGATCTAAAACTTCTGTTGCTTTTGCCA      840
  D  S  D  *

TTCCAAAACTTTGTCTTTGCCAGAAAAGTGTTGGTAACTATAAAGAAAATATATATGAAA      900

A                                                                  901
```

FIG. 1B

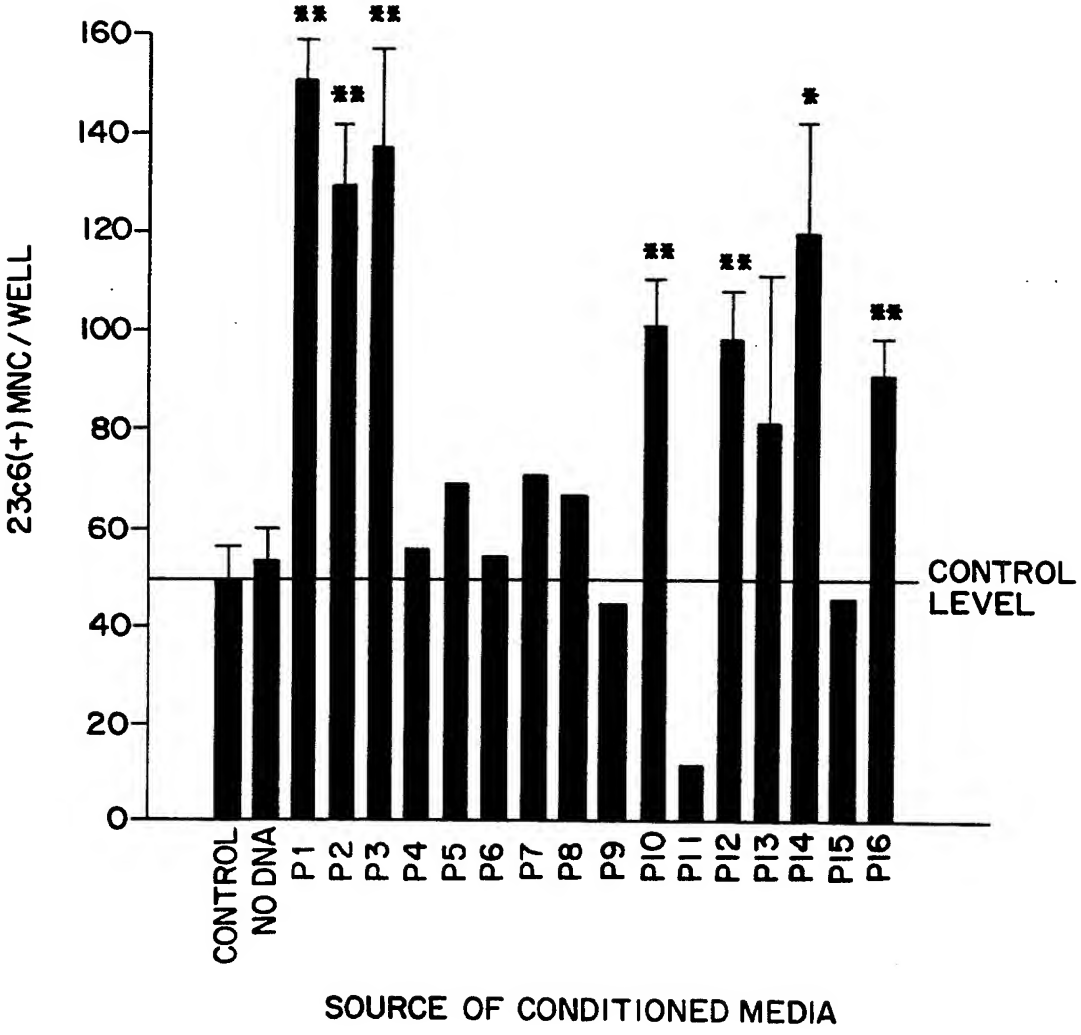
23c6(+) MNC / WELL
160
140
120
100
80
60
40
20
0
CONTROL LEVEL
CONTROL
NO DNA
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
P15
P16
SOURCE OF CONDITIONED MEDIA

FIG. 2

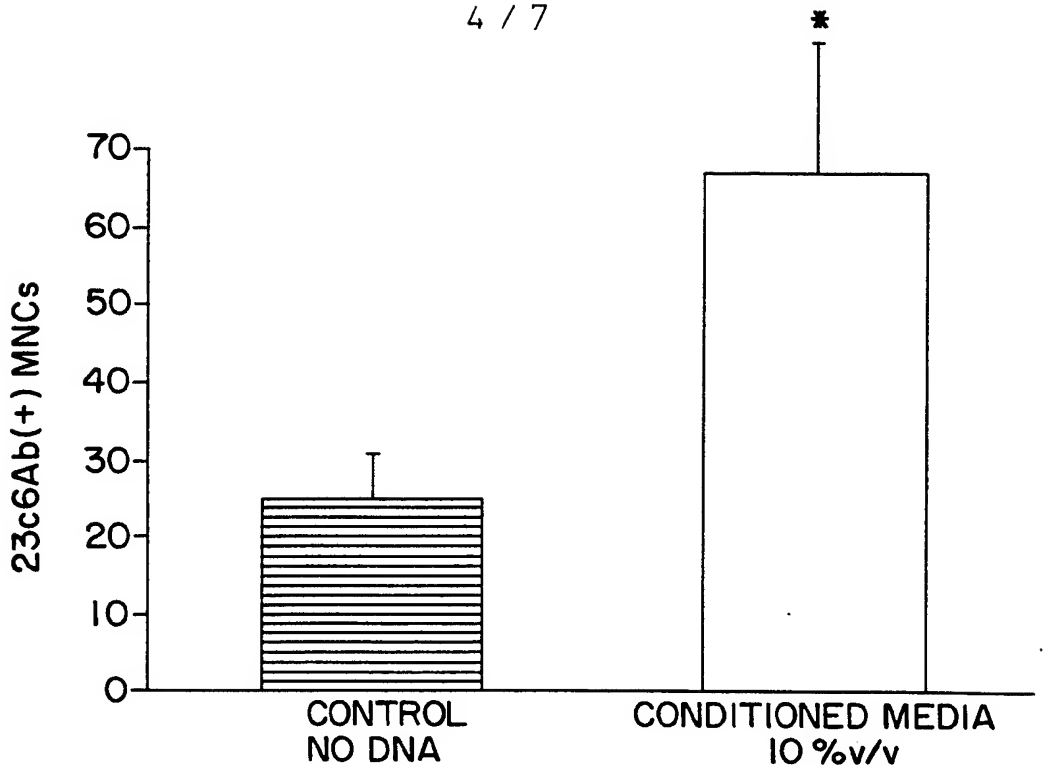
23c6Ab(+) MNCs
70
60
50
40
30
20
10
0
*
CONTROL
NO DNA
CONDITIONED MEDIA
10 %v/v

FIG. 3

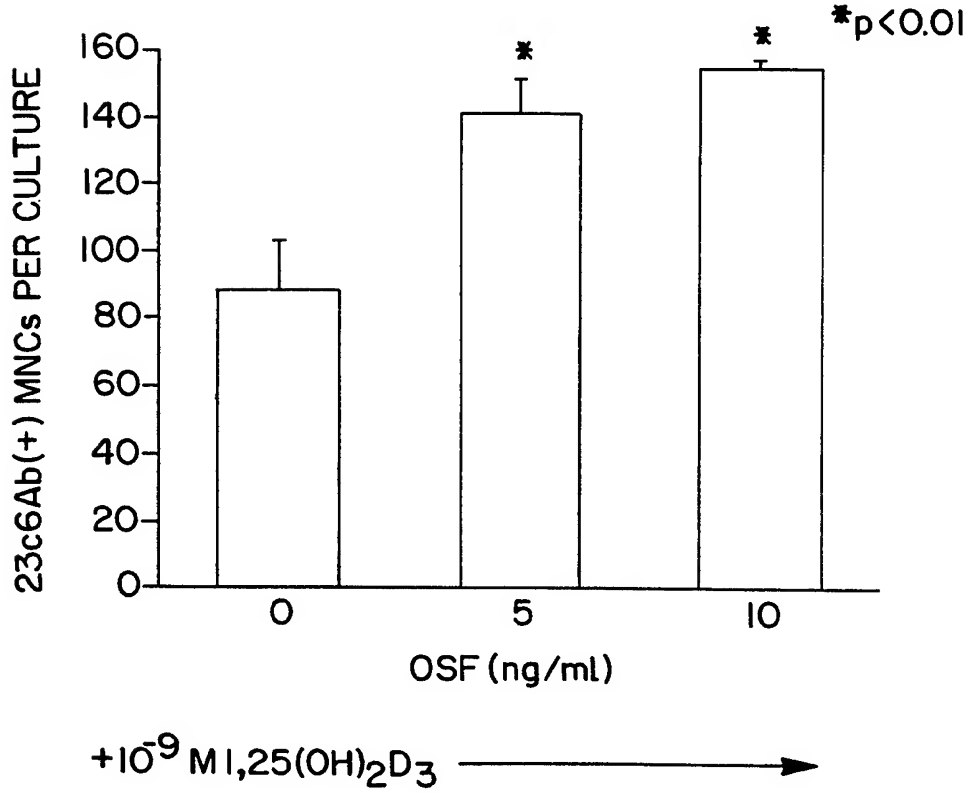
23c6Ab(+) MNCs PER CULTURE
160
140
120
100
80
60
40
20
0
*
*
*p<0.01
0
5
10
OSF (ng/ml)
+10⁻⁹ M 1,25(OH)₂D₃

FIG. 4

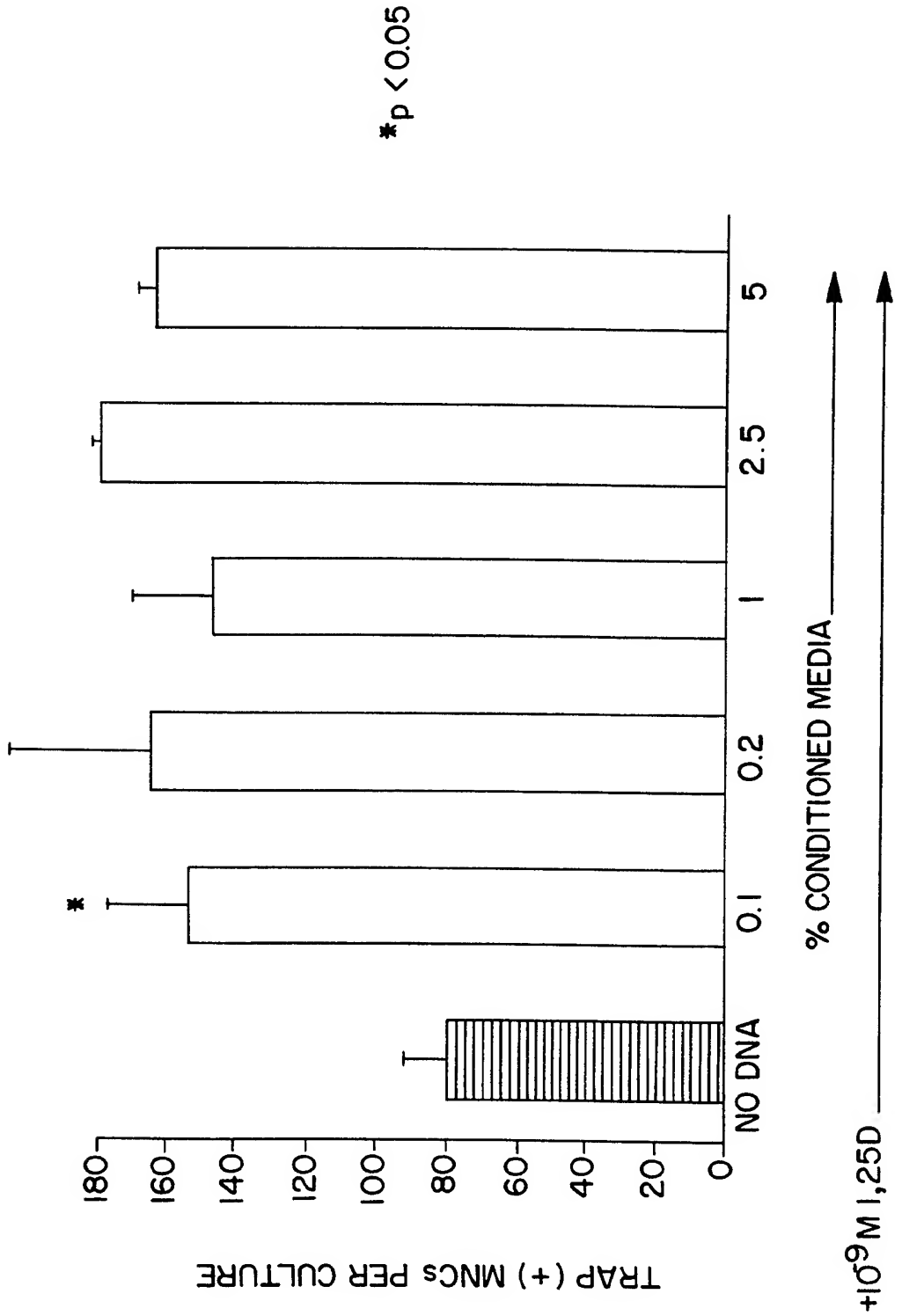
*p < 0.05
TRAP (+) MNCs PER CULTURE
180
160
140
120
100
80
60
40
20
0
NO DNA
0.1
0.2
1
2.5
5
% CONDITIONED MEDIA
+10⁻⁹M 1,25D

FIG. 5

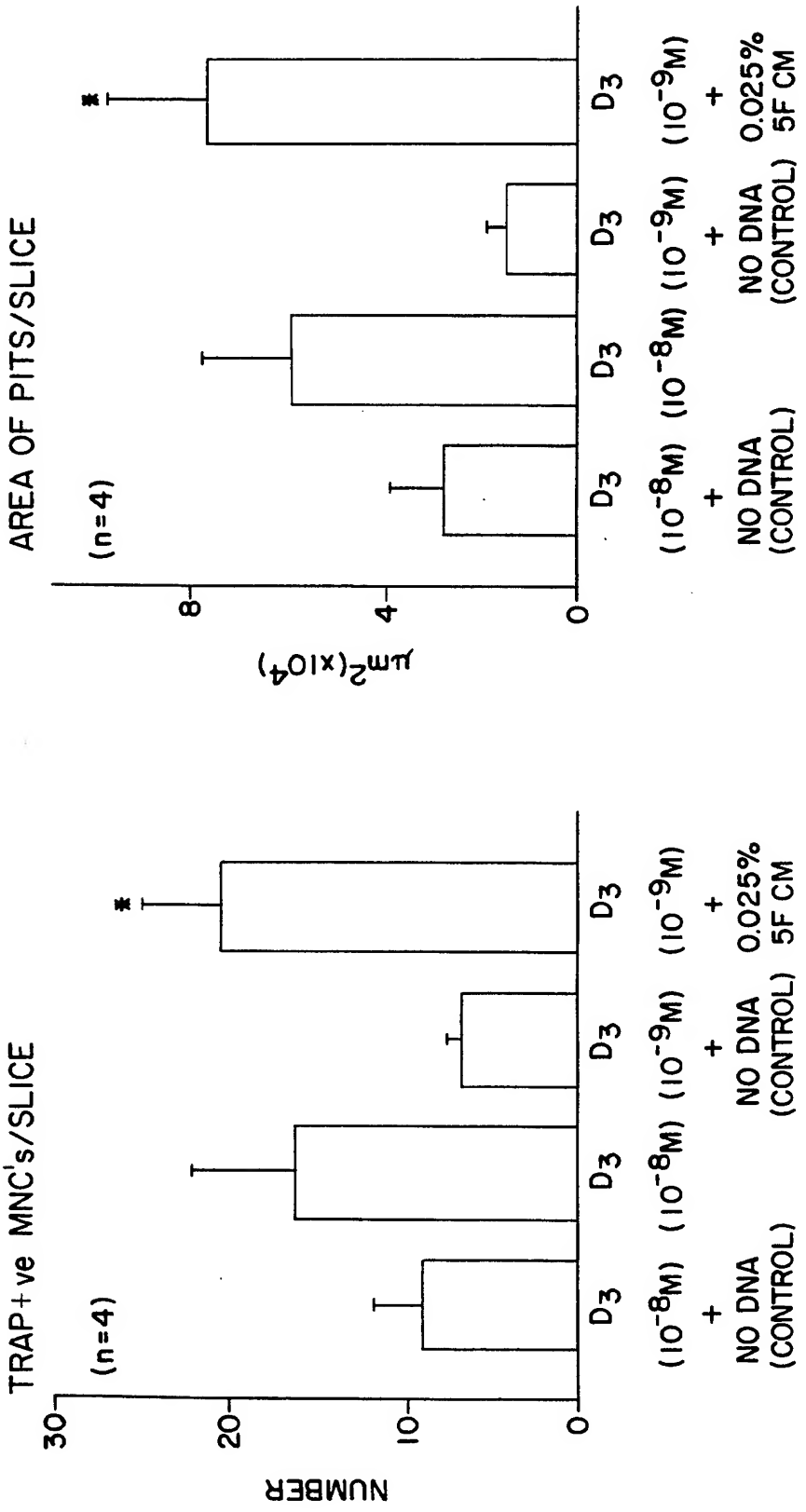

FIG. 6A

FIG. 6B

*= SIGNIFICANT INCREASE COMPARED TO $D_3(10^{-9}M)$ + No DNA (CONTROL) $p<0.05$ (STUDENT'S T-TEST)

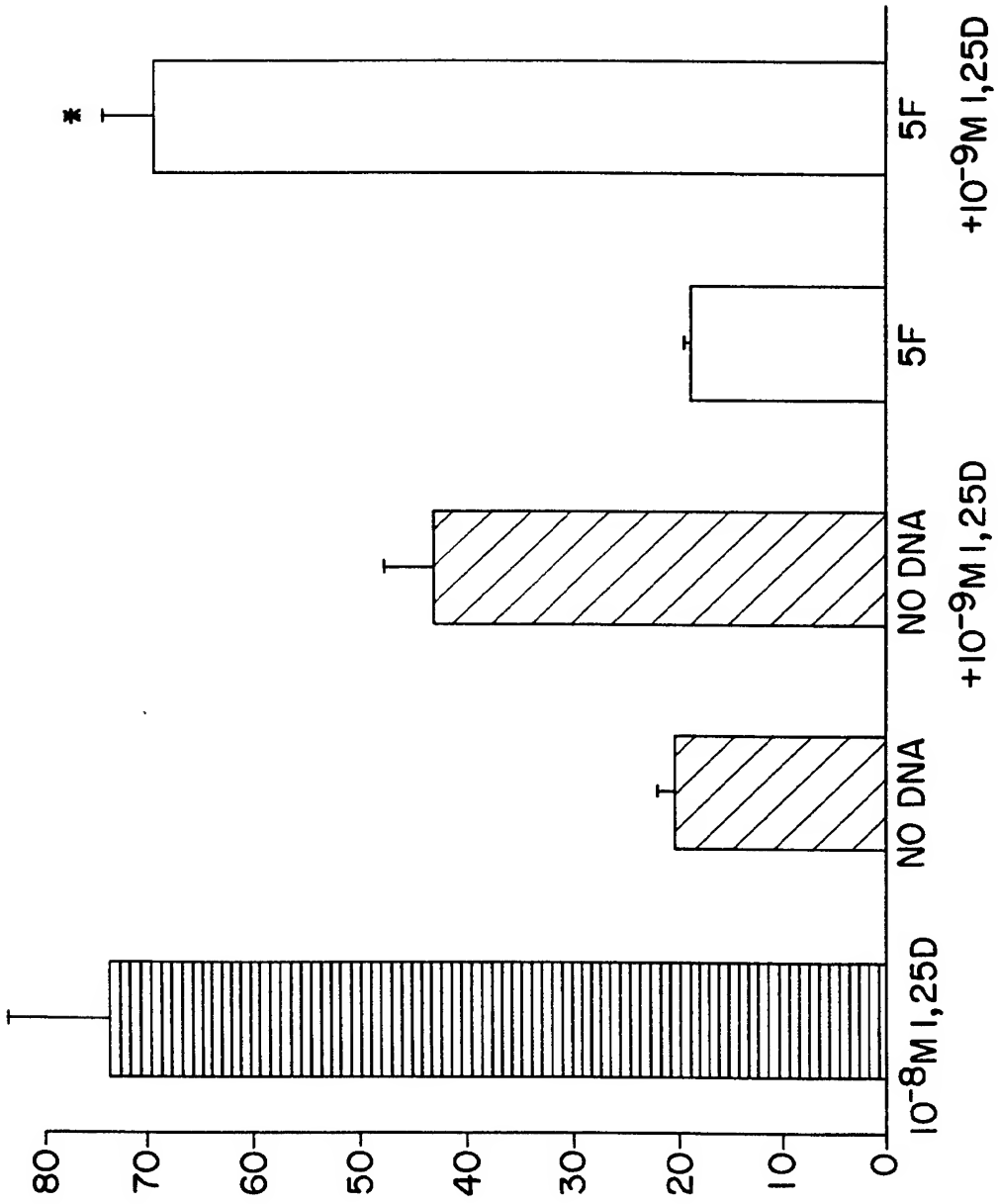
80
70
60
50
40
30
20
10
0
10^{-8}M 1,25D
NO DNA
NO DNA
+10^{-9}M 1,25D
5F
5F
+10^{-9}M 1,25D
*p<0.01, COMPARED TO NO DNA+10^{-9}M 1,25D

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09127

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5, 24.3, 24.31; 424/84, 145.1; 514/2, 8, 12; 530/350, 399; 435/69.1, 69.4, 172.3, 240.1, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 88, issued October 1991, Lee et al, "Isolation of a Murine Osteoclast Colony-Stimulating Factor", pages 8500-8504, see entire document	1-12, 17, 19
A	TRENDS IN ENDOCRINOLOGY AND METABOLISM, issued July/August 1990, G.R. Mundy, "Immune System and Bone Remodeling", pages 307-311, see pages 308-310.	1-12, 17 , 19
A	JOURNAL OF CLINICAL INVESTIGATION, Volume 67, issued May 1981, Josse et al, "Observations on the Mechanism of Bone Resorption Induced by Multiple Myeloma Marrow Culture Fluids and Partially Purified Osteoclast-Activating Factor", pages 1472-1481, see entire document.	17, 19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 AUGUST 1996	Date of mailing of the international search report 26 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PREMA MERTZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09127

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,169,837 (LAGARDE ET AL) 08 December 1992, see entire document.	1-3, 17, 19
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 269, Number 46, issued 18 November 1994, Takahashi et al, "Cloning and Identification of Annexin II as an Autocrine/Paracrine Factor That Increases Osteoclast Formation and Bone Resorption", pages 28696-28701, see entire document.	1-12, 17, 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09127

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12, 17 and 19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09127

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/11, 15/16, 15/18; C07K 14/475, 14/51, 14/47, 16/00, 16/22; A61K 38/16, 38/18

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.1, 23.5, 24.3, 24.31; 424/84, 145.1; 514/2, 8, 12; 530/350, 399; 435/69.1, 69.4, 172.3, 240.1, 252.3, 320.1

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: osteoclast growth regulating factor or osteoclast stimulating factor polypeptide, or protein or peptide, nucleic acid or DNA, production or isolation, antibody, treatment or administration or therapy.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-12, 17 and 19, drawn to a osteoclast growth regulatory factor polypeptide, DNA encoding a osteoclast growth regulatory factor polypeptide, plasmid, vector, recombinant cell, a pharmaceutical composition and method of treating osteopetrosis with a osteoclast growth regulatory factor polypeptide.
Group II, claims 13-16, and 18, drawn to an antibody and a hybridoma cell line.
Group III, claim 20, drawn to a method of treating osteoporosis with an antibody.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The products of Groups I-II do not share the same or a corresponding technical feature in that the osteoclast growth regulatory factor polypeptide, plasmid, vector, recombinant cell, a pharmaceutical composition of Group I, the antibodies and hybridoma cell line of Group II do not require each other for their practice and have separate functions all of which constitute the special technical features which define the contribution of each invention. Since these special technical features are not shared by each product and since the common features donotestablishanadvance over the prior art, the inventions of Groups I-II do not form a single inventive concept within the meaning of Rule 13.2.

The processes of Groups I and III do not share the same or a corresponding technical feature in that a method of treating osteopetrosis by administering osteoclast growth regulatory factor polypeptide of Group I, and a method of treating osteoporosis by administering antibodies of Group III, do not require each other for their practice, have separate uses and different method steps all of which constitute the special technical features which define the contribution of each invention. Since these special technical features are not shared by each process and since the common features do not establish an advance over the prior art, the inventions of Groups I and III do not share a technical relationship and do not form a single inventive concept within the meaning of Rule 13.2.